Altered receptor trafficking in Huntingtin Interacting Protein 1-transformed cells

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Summary

The clathrin-associated protein, Huntingtin Interacting Protein 1 (HIP1), is overexpressed in multiple human epithelial tumors. Here, we report that HIP1 is a novel oncoprotein that transforms cells. HIP1-transformed cells, in contrast to RasV12-transformed cells, have dysregulation of multiple receptors involved in clathrin trafficking. Examples include upregulation of the epidermal growth factor receptor (EGFR) and the transferrin receptor. Furthermore, accumulation of transferrin and EGF in the HIP1-transformed cells was increased, and breast tumors that had EGFR expressed also had HIP1 upregulated. Thus, HIP1 overexpression promotes tumor formation and is associated with a general alteration in receptor trafficking. HIP1 is the first endocytic protein to be directly implicated in tumor formation.

Introduction

The importance of altered epidermal growth factor receptor (EGFR) signaling in the generation of cancer is well documented (Kim et al., 2001). ErbB family members such as EGFR (also known as HER or ErbB1) and ErbB2 (also known as HER2) are overexpressed in multiple spontaneously occurring human cancers. Moreover, overexpression of either EGFR (Di Fiore et al., 1987a) or erbB2 (Di Fiore et al., 1987b) transforms NIH/3T3 fibroblasts. In addition, downstream signaling targets of the EGFR, including *Ras* and *Raf*, have been found to have activating mutations in a variety of human cancers (Bos, 1989; Davies et al., 2002). The net effect of these changes is to lead to inappropriate activation of mitogenic signaling pathways, including the extracellular signal-regulated kinase (ERK) pathway with resultant cellular transformation.

One mechanism whereby cells regulate intracellular signaling is via clathrin-mediated growth factor receptor trafficking. By this process, cellular proteins that regulate endocytosis also modulate the signal transduction pathways that originate from growth factor receptors (Di Fiore and De Camilli, 2001; Vieira et al., 1996). Although dysregulation of proteins involved in receptor trafficking may lead to abnormal intracellular signaling and cancer in principle, none of these proteins have yet been found to have oncogenic properties in vivo.

The Huntingtin Interacting Protein 1 (HIP1) has been shown to be a cofactor in clathrin-mediated endocytosis (Legendre-Guillemin et al., 2002; Metzler et al., 2001; Mishra et al., 2001; Rao et al., 2001; Waelter et al., 2001). HIP1 specifically associates with and colocalizes with clathrin and the adaptor protein AP-2. In addition, a phosphatidylinositol lipid binding epsin N-terminal homology (ENTH) domain is present at the N terminus of HIP1. To date, this domain has only been found in proteins that participate in clathrin-mediated trafficking. However, the specific function of HIP1 in endocytosis has not yet been delineated.

Recently, we demonstrated that expression of the HIP1 protein is elevated in multiple primary human epithelial cancers. Increasing HIP1 expression is correlated with increasing aggressiveness of prostate cancer, and HIP1 expression is an independent predictor of relapse in patients with prostate cancer (Rao et al., 2002). In addition, expression of HIP1 in prostate tumors from TRAMP mice was higher by at least an order of magnitude than normal mouse prostate tissue. These results suggested the possibility that HIP1 overexpression may promote the transformation of normal cells into cancer cells. Here, we demonstrate that HIP1 transforms NIH/3T3 fibroblasts and upregulates

SIGNIFICANCE

The upregulation of growth factor receptors, such as those for epidermal growth factor, has been implicated as a mechanism of transformation in multiple human cancers. In addition to mutations, such as gene amplification, leading to elevated levels of EGFR, alterations in receptor trafficking have been shown to alter receptor levels and intracellular signal transduction, and hypothesized to lead to oncogenesis. Here, oncogenic transformation is shown as a direct result of the overexpression of Huntingtin Interacting Protein 1 (HIP1), a protein that is involved in clathrin-mediated trafficking. Furthermore, overexpression of HIP1 led to changes in the total levels of receptors and to altered trafficking of EGF and transferrin. These results implicate receptor-mediated trafficking as a mechanism whereby oncogenic transformation may occur or be sustained by decreasing the dependence of transformed cells on growth factors.

multiple receptors involved in trafficking, including the well-studied EGFR. To begin to study the mechanism of this transformation, the effects of the EGFR upregulation were tested in detail to provide an example for how upregulation of growth factor receptors via HIP1 overexpression could induce and/or maintain the transformed phenotype. In addition, we report that EGFR expression in primary breast tumors coincides with HIP1 overexpression.

Results

As a next step in our studies of HIP1 and its role in tumorigenesis, we tested if HIP1 had oncogenic properties. We established NIH/3T3 cell lines that stably overexpressed HIP1 under the control of the CMV promoter. Every hygromycin-resistant clone isolated demonstrated overexpression of HIP1, suggesting that HIP1 overexpressing cells had a selective advantage over cells that did not overexpress HIP1. In a parallel experiment, we infected NIH/3T3 cells with retrovirus expressing HIP1 under the control of the LTR promoter and isolated G418-resistant clones, which were, again, all overexpressing HIP1. On the other hand, infection with a dominant interfering proapoptotic HIP1 mutant, designated HIP1/ΔE (Rao et al., 2002), did not produce any clones expressing HIP1/ΔE, except for one clone with extremely low levels of expression (Figure 1).

For the purposes of our analyses, we studied cell lines with moderate overexpression of HIP1 (designated 3T3/HIP1^{mod}) and high overexpression of HIP1 (designated 3T3/HIP1^{hi}). In addition, three independent clones derived from transfection with vector alone (designated 3T3/Ctrl) and three clones that expressed high levels of oncogenic RasV12 (designated 3T3/Ha-RasV12) were selected as negative and positive controls, respectively. The overexpressed human HIP1 protein was readily distinguished from endogenous mouse HIP1 using the human HIP1-specific monoclonal antibody, HIP1/4B10. The levels of total HIP1 were compared using a BioRad Fluor S max camera and the monoclonal antibody, HIP1/1B11, which recognizes both human and mouse HIP1 (Figure 2A, lanes 1–3 versus lanes 4–9). 3T3/HIP1^{hi} cell lines expressed 100- to 200-fold more HIP1 (while 3T3/HIP1^{mod} expressed 5–50 fold more HIP1 than endogenous murine HIP1) as compared to vector transfected 3T3 cells that only expressed endogenous murine HIP1 (Figure 2B).

After establishing these cell lines, the effect of HIP1 on cellular proliferation rates was tested under various growth conditions. All cell lines expressing HIP1 at moderate and high levels had higher growth rates and grew to greater densities as compared to control cell lines under standard growth conditions (Figure 2C). As expected, cell lines with overexpression of oncogenic Ras also grew faster and to higher saturation densities (Figure 2C). Surprisingly, 3T3/HIP1^{hi} cells were able to grow in medium supplemented with only 0.1% fetal bovine serum (FBS), whereas 3T3/Ctrl, 3T3/HIP1^{mod}, and 3T3/Ha-RasV12 cell lines did not significantly proliferate under these conditions (Figure 2D). Moreover, 3T3/HIP1^{hi} cell lines were able to grow to a higher density by 8 days than 3T3/Ctrl cell lines under both normal and low-serum conditions (Figure 2E).

Exposure of NIH/3T3 cells to medium containing 0.1% FBS for 48 hr is sufficient to cause exit from the cell cycle and arrest in G0 (Jones and Kazlauskas, 2001). Given the ability of 3T3/HIP1^{hi} cells to grow in medium with 0.1% FBS, we assayed to determine if there were abnormalities in the cell cycle status of cell lines overexpressing HIP1. When assayed by propidium iodide staining and FACS analysis, a larger proportion of the 3T3/HIP1^{hi} cells were found to be in the S phase of the cell cycle (44.9 ±10.2%), compared to control cell lines (19.7% ± 2.3%), when grown to 90% confluence under normal growth conditions (Figure 3A). As expected, similar results were seen with 3T3/Ha-RasV12 cells (data not shown). When the experiment was repeated with cells grown for 48 hr in medium with 0.1% FBS, 3T3/HIP1^{hi} cell lines still had a significantly higher proportion of cells in the S phase of the cell cycle (27 ± 2.8% for 3T3/HIP1^{hi} versus 11.2 ± 2.6% for 3T3/Ctrl), indicating increased progression through the cell cycle (Figure 3B). These data, together with the ability to grow in 0.1% FBS, indicate that drastic reductions in the amounts of growth factors in the medium had a minimal effect on the ability of 3T3/HIP1^{hi} cells to progress through the cell cycle. In the complete absence of serum, HIP1-overexpressing cells survived but did not proliferate, whereas control cells did not survive (data not shown). This suggests that HIP1 overexpression increased the sensitivity of

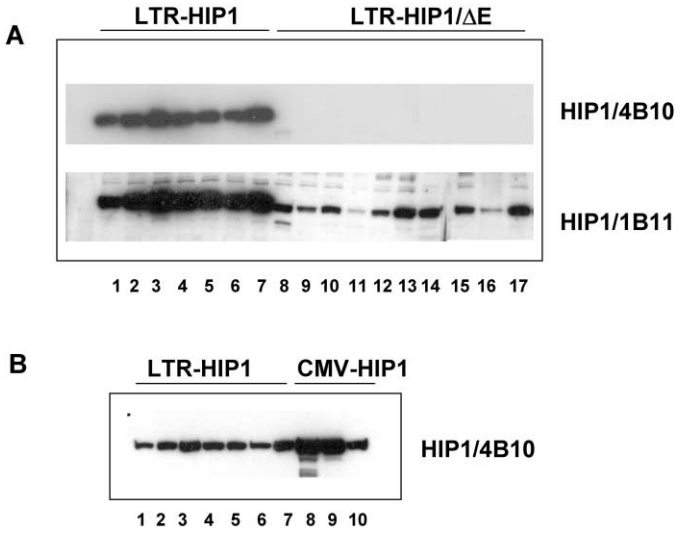

Figure 1. Stable overexpression of wild-type HIP1 but not HIP1/ΔE was found in all selected clones

A: Western blot analysis of cell lines with overexpression of HIP1 under the control of the LTR promoter. MSCV-neo/HIP1 was constructed by subcloning the full-length HIP1 cDNA from pcDNA3/HIP1 into the MSCV-neo vector. MSCV-neo/HIP1/ΔE was constructed by subcloning a fragment of HIP1 lacking the ENTH domain from the pcDNA3HIP1/ΔE into MSCV-neo (Rao et al., 2002). These constructs have HIP1 under the control of the LTR promoter. NIH/3T3 cells were infected with retroviral supernatants obtained from transfection of the MSCV-neo constructs into 293T cells as previously described (Saint-Dic et al., 2001). Following transfection, cells were selected in G418 (400 μg/ml) for 2 weeks, and individual clones were isolated and subsequently expanded. Extracts prepared from these clones were loaded onto gels as follows: Lanes 1–7, clones from transfection with MSCV-HIP1; lanes 8–17, clones from transfection with MSCV-neo/HIP1/ΔE. Note that in lane 8, there is a low-intensity band smaller than full-length HIP1, which crossreacts with both the anti-HIP1 monoclonal antibodies and was most likely the HIP1/ΔE protein product.

B: Comparison of HIP1 expression levels in cell lines generated using the MSCV-neo/HIP1 (LTR-HIP1) construct and the pTRE2-hyg/HIP1 construct (CMV-HIP1). Gels were loaded as follows: Lanes 1–7, LTR-HIP1-expressing cells as in **A**; lanes 8–9 were loaded with 3T3/HIP1^{hi}; lane 10 was loaded with 3T3/HIP1^{mod}.

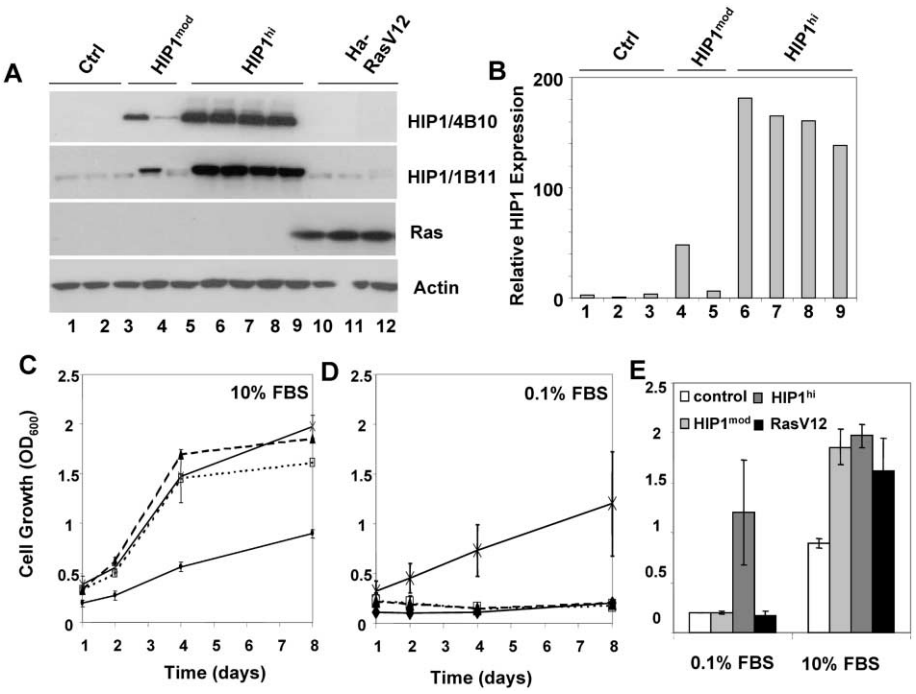

Figure 2. HIP1 overexpression leads to altered growth of NIH/3T3 cells

A: Western blot analysis of NIH/3T3 cell lines used in this study. Cell lines were generated by transfection of constructs as described (Saint-Dic et al., 2001). Gels were loaded as follows: Lane 1–3, clones 3T3/4.1B, 3T3/4.2.B, and 3T3/4.3.E (3T3/ctrl); Lanes 4–5, clones 3T3/5.2.A and 3T3/5.3.E (3T3/HIP1^{mod}); Lanes 6–9, clones 3T3/5.1.E, 3T3/5.2.D, 3T3/5.2.C, and 3T3/5.3.F (3T3/HIP1^{hi}); Lanes 10–12, clones 3T3/6.1A, 3T3/6.1.E, and 3T3/6.3.A (3T3/Ha-RasV12). 20 μg of protein was subjected to standard Western blot analysis with the anti-HIP1 monoclonal antibodies, HIP1/4B10 and HIP1/1B11 (Rao et al., 2002). 50 μg of protein was subjected to Western blot analysis with a monoclonal Ras antibody, and actin immunoblotting was used to demonstrate equal loading of lanes.

B: Quantitation of relative protein levels in HIP1-overexpressing cells. HIP1 immunoblots were developed with Pico Plus ECL reagent (Pierce), and quantitated using a BioRad Fluor S Max camera (BioRad Corporation). Multiple dilutions of the samples were used to confirm relative levels of expression (not shown). Bars 1–9 correspond to lanes 1–9 in **A**.

C–E: The growth of 3T3/Ctrl, 3T3/HIP1^{mod}, 3T3/HIP1^{hi}, and 3T3/Ha-RasV12-expressing cells was measured by an MTT assay following manufacturer's instructions (Roche Molecular Biochemicals). Cell lines were grown in 96-well plates in growth media supplemented with either 10% FBS **(C)** or 0.1% FBS **(D)** for 1, 2, 4, and 8 days. ●, 3T3/Ctrl; ▲, 3T3/HIP1^{mod}; X, 3T3/HIP1^{hi}; and □, 3T3/Ha-RasV12). Measurements were made in quadruplicate for each of the cell lines (see **A**), and the experiments were repeated twice using the MTT measurements and one additional time by counting cells with a hemacytometer. **E:** Relative density of the cell lines after 8 days of growth in either 10% FBS or 0.1% FBS.

cells to growth factors. As expected from their inability to proliferate in 0.1% FBS, 3T3/HIP1^{mod} cells did not have as great an increase in the proportion of cells in S phase of the cell cycle (Figure 3B).

Since abnormalities in proliferation are a hallmark of cancerous cells and HIP1 is overexpressed in tumor cells (Rao et al., 2002), these data suggested that HIP1 expression may be directly oncogenic. HIP1-overexpressing cell lines were therefore assayed for anchorage-independent growth in soft agar (Di Fiore et al., 1987a), ability to form foci when plated at low density (Sage et al., 2000), and ability to form tumors in nude mice (Kokai et al., 1989). By all three counts, HIP1-overexpressing cells were transformed, while control cell lines were not (Figures 3C–3E).

Both 3T3/HIP1^{hi} and 3T3/HIP1^{mod} cell lines formed colonies in soft agar (Figure 3C). 3T3/HIP1^{mod} cells formed fewer colonies, and colonies of a smaller size, than 3T3/HIP1^{hi} cell lines (Figure 3C, panel 2 versus panel 3). Additionally, the clone with the lowest level of expression of HIP1, designated 3T3/5.3.E, displayed the slowest rate of growth in soft agar (data not shown). Equivalent numbers of 3T3/HIP1^{hi} cells formed more colonies in soft agar than mutant Ras-expressing cells. Colonies formed by mutant Ras-expressing cells tended to be larger on average than colonies formed by HIP1^{hi} cells. In addition, stable cell lines generated by retroviral infection with MSCVneoHIP1 that uses the LTR promoter (Figure 1) were also able to form colonies in soft agar (data not shown). HIP1-overexpressing lines were also able to form foci when plated a low density (1.3×10^3 cells/100 mm plate), whereas control cell lines were not (Figure 3D). The number of foci formed correlated with the level of HIP1 expression, with fewer colonies formed by 3T3/HIP1^{mod} cells compared to the 3T3/HIP1^{hi} cells. As in the soft agar assay, the number of colonies seen with RasV12-overexpressing cell lines was lower than that seen with 3T3/HIP1^{hi} cells.

Finally, the ability of these cell lines to form tumors in vivo was addressed by subcutaneous injection of HIP1-overexpressing cell lines into nude mice. All 3T3/HIP1^{hi} cell lines formed tumors in nude mice at every injection site (Figures 3E and 4). Neither the control cell lines nor 3T3/HIP1^{mod} cell lines formed tumors in nude mice during the 16-day course of the experiment. It is quite possible that 3T3/HIP1^{mod} cell growth in nude mice, similar to growth in soft agar, lags behind that of 3T3/HIP1^{hi} cells, and these lines would have eventually formed tumors. Cells expressing RasV12 were used as positive controls in the nude mouse assays and formed the largest tumors (Figure 4). Since these transformation assays all measure different aspects of tumorigenesis and mutant Ras-transformed cells behaved differently from the HIP1-transformed cells in several of these assays, it is likely that HIP1-mediated transformation affects different aspects of tumorigenesis, depending on its level of overexpression, and is distinct from RasV12-mediated transformation.

The observation of the ability of HIP1-overexpressing cells to grow in the presence of limiting serum suggested to us that these lines might have increased activation of receptor tyrosine kinase signaling pathways. This is a well-known mechanism for transformation which abrogates the requirement for growth factors in the medium in both hematopoietic (Daley and Baltimore, 1988) and nonhematopoietic cancers (Di Fiore et al., 1987b). Such inappropriate activation of intracellular signaling can occur either by mutation or simple overexpression of a receptor tyrosine kinase (Di Fiore et al., 1987a) where elevated

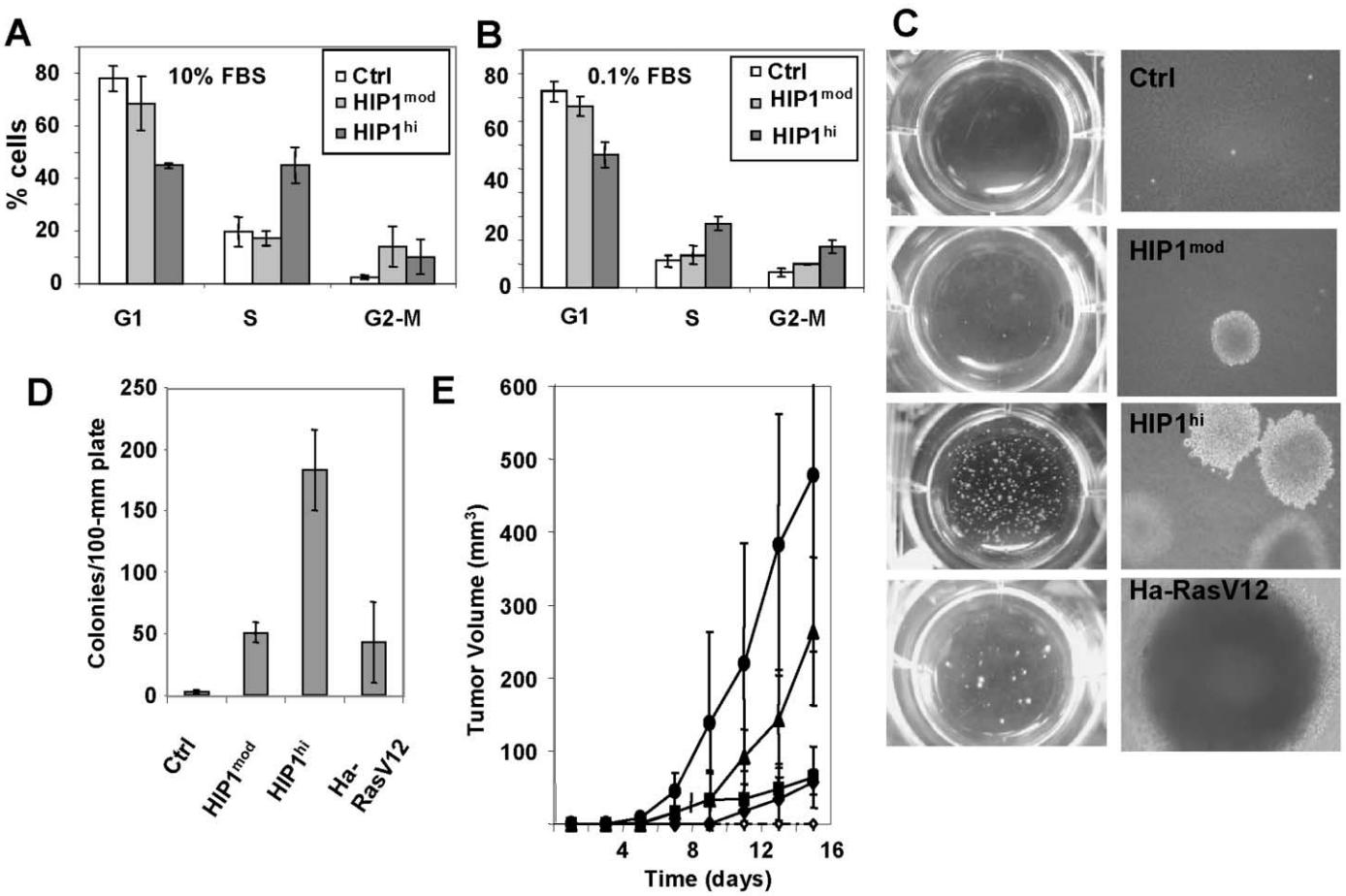

Figure 3. HIP1 overexpression transforms NIH/3T3 cells

A–B: Cell cycle analysis of 3T3/HIP1 stable lines grown in normal medium **(A)** and in growth medium supplemented with only 0.1% FBS **(B)**. 10^6 cells were plated on 100 mm dishes, grown as described (in 10% or 0.1% FBS for 24 hr), trypsinized, fixed with 70% ethanol, and stained with propidium iodide (Sigma) to determine DNA content and therefore cell cycle status by flow cytometry.

C: Anchorage-independent growth of 3T3 stable cell lines in soft agar. Cell lines at the same passage number were used for these assays, which have been previously described (Di Fiore et al., 1987a). Shown are photographs of representative wells for 3T3/Ctrl, 3T3/HIP1^{mod}, 3T3/HIP1^{hi}, and 3T3/Ha-RasV12 cell lines (left panels) to indicate the frequency of colonies formed in soft agar, and photomicrographs (100×) of the same wells to show colony size (right panels) after 16 days in culture. Note that the colony shown in the photomicrograph for 3T3/Ha-RasV12 is larger than the photographic field. The central dark area is the densest portion of this colony, and the surrounding lighter area shows individual cells that form part of the colony. The light flecks shown in the photomicrograph for 3T3/Ctrl are individual cells, unable to form the colonies as shown for 3T3/HIP1^{hi} and 3T3/HIP1^{mod}. Three independent experiments yielded similar results.

D: Proliferation of 3T3 stable lines after plating at low density. 1,300 cells were plated as described (Sage et al., 2000). This experiment was done in duplicate, and shown are the mean number of colonies and standard deviation for 3T3/Ctrl (n = 3 cell lines), 3T3/HIP1^{mod} (n = 2 cell lines), 3T3/HIP1^{hi} (n = 4 cell lines), and 3T3/Ha-RasV12 (n = 3 cell lines), after 14 days in culture.

E: Growth of 3T3 stable lines in nude mice. 5×10^5 cells of each cell line were injected subcutaneously into the rear flanks of 6–8 week old Ncr athymic nude mice (Taconic Farms) and the growth of the tumors was measured every other day. The volume of the tumors was calculated by the formula $V = (L \times W^2)/2$, where L is the length and W is the width of the tumor. For each experimental cell line (3T3/HIP1^{mod} and 3T3/HIP1^{hi}), 3 sites were injected, while each mouse received a shoulder injection of a positive control (3T3/6.1.E, a RasV12-overexpressing cell line) and a negative control (3T3/4.2.B, a 3T3/Ctrl cell line). Results are means and standard deviations for each cell line (■, 3T3/5.1.E; ▲, 3T3/5.2.D; ●, 3T3/5.2.C; ◆, 3T3/5.3.F; ◇, 3T3/4.2.B).

levels of growth factor receptors can compensate for lower amounts of growth factors in the surrounding tissue fluids. Given that HIP1 is overexpressed in multiple epithelial cancers and its overexpression-transformed NIH/3T3 cells, we began our analysis of the effect of HIP1 overexpression on growth factor receptors by testing the well-studied EGFR. Remarkably, all 3T3/HIP1^{hi} cell lines showed increased levels of EGFR (Figure 5A, panel I; lanes 4–7). In addition, Western blot analysis for phosphorylated forms of the EGFR showed that the EGFR was phosphorylated at multiple tyrosine residues, indicating that the receptor was not only more highly expressed but also activated under normal conditions (10% FBS) of growth (Figure 5A, panels II–V). Flow-cytometric analysis indicated that surface levels of EGFR were also elevated in 3T3/HIP1^{hi} cell lines (Figure 5B). The same upregulation of EGFR was not seen in 3T3/HIP1^{mod} cells (data not shown) or the RasV12-transformed cells.

Phosphorylation of the EGFR leads to activation of several intracellular signaling cascades, including the activation of the ERK, PtdIns 3-kinase, and PLCγ pathways (Figure 6). In 3T3/HIP1^{hi} cell lines, the entire ERK cascade was found to be acti-

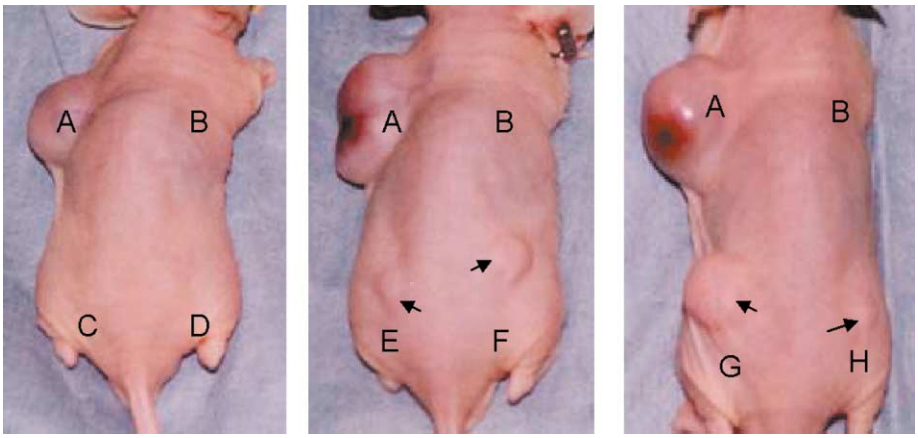

Figure 4. Photographs of nude mice 2 weeks after subcutaneous injection of HIP1-overexpressing cell lines

Injection sites for the various cell lines are listed below. **A:** Positive Control (3T3/Ha-RasV12); **B:** Negative control (3T3/Ctrl); **C:** 3T3/5.3.E (3T3/HIP1^{mod}); **D:** 3T3/5.2.A (3T3/HIP1^{mod}); **E:** 3T3/5.1.E (3T3/HIP1^{hi}); **F:** 3T3/5.2.D (3T3/HIP1^{hi}); **G:** 3T3/5.2.C (3T3/HIP1^{hi}); **H:** 3T3/5.3.F (3T3/HIP1^{hi}).

vated, with hyperphosphorylation of Raf, MEK-1/2, ERK-1/2, and the downstream target of ERK, p90Rsk (Figure 5C; compare lanes 4–7 with lanes 1–3 in all panels). Total levels of ERK and Raf proteins were unchanged. Thus, HIP1 overexpression contributes to the activation of a well-characterized mitogenic signaling pathway, via upregulation and activation of EGFR. We also found that PtdIns 3-kinase, but not PLCγ, was tyrosine phosphorylated in the 3T3/HIP1^{hi} cell lines growing in 10% FBS (data not shown). In contrast, none of the control cell lines had activation of any of these pathways. As expected, 3T3/Ha-RasV12 cell lines did have the activation of the MAPK pathway (Figure 5C; compare lanes 8–10 with lanes 1–3 in all panels), but not of PtdIns 3-kinase (data not shown).

There was a striking correlation of the expression levels of HIP1 with growth characteristics and the results of the transformation assays (Table 1). Transformation in 3T3/HIP1^{hi} cell lines was more robust than that seen in 3T3/HIP1^{mod}, as the latter did not form tumors in nude mice and formed fewer colonies in soft agar. This is very interesting as there is a strong correlation between HIP1 expression levels and tumor progression in pros-

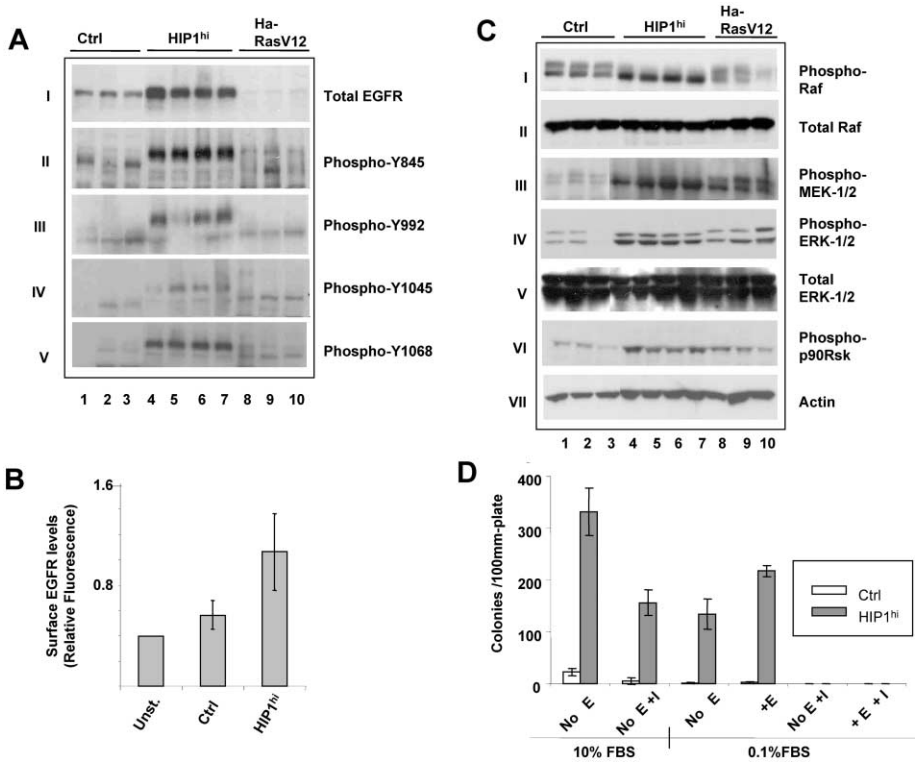

Figure 5. HIP1-transformed NIH/3T3 cells have elevated EGFR levels

A: Increase in EGFR levels and EGFR tyrosine phosphorylation in HIP1-overexpressing lines. Gels were loaded as follows: lanes 1–3, clones 3T3/4.1B, 3T3/4.2.B, and 3T3/4.3.E (3T3/Ctrl); lanes 4–7, clones 3T3/5.1.E, 3T3/5.2.D, 3T3/5.2.C, and 3T3/5.3.F (3T3/HIP1^{hi}); lanes 8–10, clones 3T3/6.1A, 3T3/6.1.E, and 3T3/6.3.A (3T3/Ha-RasV12). Phosphotyrosine residue-specific antibodies were used to immunoblot identically loaded gels. Phospho-Y845 is a potential Src-phosphorylation site, while the other phosphotyrosine residues are autophosphorylation sites.

B: Flow cytometric analysis of intact 3T3/Ctrl and 3T3/HIP1^{hi} cells with an extracellular domain-specific phycoerythrin-conjugated EGFR antibody shows that 3T3/HIP1^{hi} cells had higher levels of surface EGFR, as detected by fluorescence intensity. The average fluorescence intensities and standard deviations for control and 3T3/HIP1^{hi} cells are shown. Stably transfected cell lines were grown to 90% confluence and detached from plates with 0.25% trypsin, and 10^6 cells were labeled with PE-conjugated monoclonal EGFR antibody per manufacturer's directions (Pharmingen, San Diego, CA) and analyzed on a Beckman-Coulter Elite.

C: Western blot analysis of the Raf-MEK-ERK mitogenic signaling cascade. Gels were loaded in the same order as in (**A**), and Western blot analysis was carried out as described.

D: Proliferation of 3T3 stable lines after plating at low density in 10% or 0.1% FBS, EGF, and/or the EGFR inhibitor CI-1033. All cells were supplemented with 0.1% FBS or 10% FBS as indicated. E = 100 ng/ml EGF; I = 1 μM CI-1033. 1,300 cells were plated on 100 cm dishes as described (Sage et al., 2000). This experiment was performed twice with the same results, showing that the EGFR inhibitor significantly inhibited formation of colonies in 10% FBS ("no E +I" compared to "no E") and that no colonies formed in the control cells (3T3/Ctrl) grown in 0.1% FBS ("no E") or HIP1-transformed cells grown in the presence of the EGFR inhibitor ("No E +I" or "+E +I").

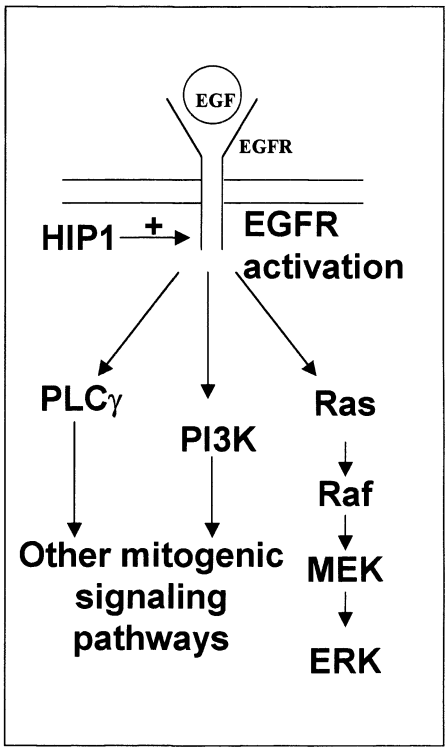

Figure 6. Schematic of EGFR-stimulated pathways

Binding of EGF to EGFR results in activation of several mitogenic signaling pathways. Our results show activation of the Raf-MEK-ERK signaling cascade in 3T3/HIP1^{hi} cells and RasV12-overexpressing cells. In addition, the EGFR and PtdIns 3-kinase are constitutively tyrosine-phosphorylated in the HIP1 overexpressing cells but not the RasV12-overexpressing cells. Abbreviations, PLCγ, phospholipase Cγ; PI3K, phosphatidylinositol 3-kinase.

tate cancer, with higher levels of HIP1 found in more aggressive tumors (Rao et al., 2002). Thus, it was possible to recapitulate the growth and survival advantages of high-level HIP1 expression in tumors by direct overexpression of different levels of the HIP1 protein in NIH/3T3 fibroblasts.

It is also of interest to note that the upregulation of the EGFR in 3T3/HIP1^{hi} cells was associated with both tumor growth in nude mice and growth in limiting serum, while activation of the ERK pathway in the absence of EGFR activation in RasV12-expressing cells correlated with tumor growth in nude mice, but not serum-independent growth (Table 1). Since EGFR is known to activate pathways other than the ERK cascade, such as PtdIns 3-kinase and PLCγ (Yarden and Sliwkowski, 2001), these pathways may be required for proliferation under low-serum conditions. The tyrosine phosphorylation of PI3K in 3T3/HIP1^{hi} cells may account for this phenomenon. The fact that we could not detect PLCγ tyrosine phosphorylation could be due to compensating dephosphorylation in these cells. The data on PtdIns 3-kinase activation, together with the fact that these other pathways were not activated by RasV12 expression, predict that the RasV12-transformed cells would depend on serum for growth (Table 1 and Figure 5). In addition, the lack of activation of EGFR in 3T3/HIP1^{mod} cells did not abrogate their ability to grow to higher densities than control cells or to grow in soft agar. This indicates that HIP1 overexpression may affect multiple pathways that are transforming.

These provocative data led to the question of whether the HIP1-transformed phenotype was cell intrinsic, or required the presence of a single growth factor, such as EGF, or a combination of growth factors in the extracellular milieu. In addition, since the effect of HIP1 was likely more widespread than just the EGFR and the transferrin receptor, we wanted to test if EGFR-mediated signaling was the only pathway that contributed to the mechanism of HIP1-mediated transformation. To determine if the transformed phenotype was cell-autonomous, the soft agar assay was repeated with medium supplemented with 0.1% FBS rather than the standard of 10% FBS. Under these conditions, 3T3/HIP1^{hi} cells failed to form anchorage-independent colonies (data not shown), indicating that supplementation with FBS was required for anchorage-independent growth. On the other hand, 3T3/HIP1^{hi} cells do proliferate when plated in medium supplemented with 0.1% FBS, albeit at a much slower rate than in 10% FBS (Figure 2D). This led to the hypothesis that these cells were secreting an EGF-like substance or other growth factors into the media that supported cellular growth in low serum concentrations. In order to test this, we utilized conditioned medium from 3T3/HIP1^{hi} cells to supplement the growth medium for 3T3/Ctrl cells (Figure 7B). This medium failed to stimulate 3T3/Ctrl cells to proliferate at an increased rate. This result could be due to the fact that 3T3/Ctrl cells did not have large amounts of EGFR (Figure 5A). To rule out this possibility, we tested the ability of conditioned medium to supplement the growth medium of MCF10A cells, finding that there was little effect on their growth (Figure 7A). MCF10A cells are exquisitely sensitive to EGF or other peptide hormones with EGF-like activity, and have been used as a standard for testing for EGF-like activity in conditioned medium (Ethier et al., 1991). Since conditioned medium from 3T3/HIP1^{hi} cells failed to stimulate growth of MCF10A cells, we concluded

Table 1. Summary of growth and transformation properties of cells used in this study

	Growth		Transformation assays			Signal transduction		
	10% FBS	0.1% FBS	S.A.	L.D.P.	Nude mouse	EGFR	ERK cascade	PI-3-K
3T3/Ctrl	+	−	−	−	−	−	−	−
3T3/HIP1mod	++[1]	−	+	+	−	−	−	−
3T3/HIP1hi	++[1]	+	+	+	+	+	+	+
3T3/Ha-RasV12	++[1]	−	+	+	+	−	+	−

Abbreviations: FBS, fetal bovine serum; S.A., soft agar assay for anchorage-independent growth; L.D.P, low density plating assay for transformation; PI-3-K, PtdIns 3-kinase tyrosine phosphorylation.
[1] ++ represents growth to higher density than control cells.

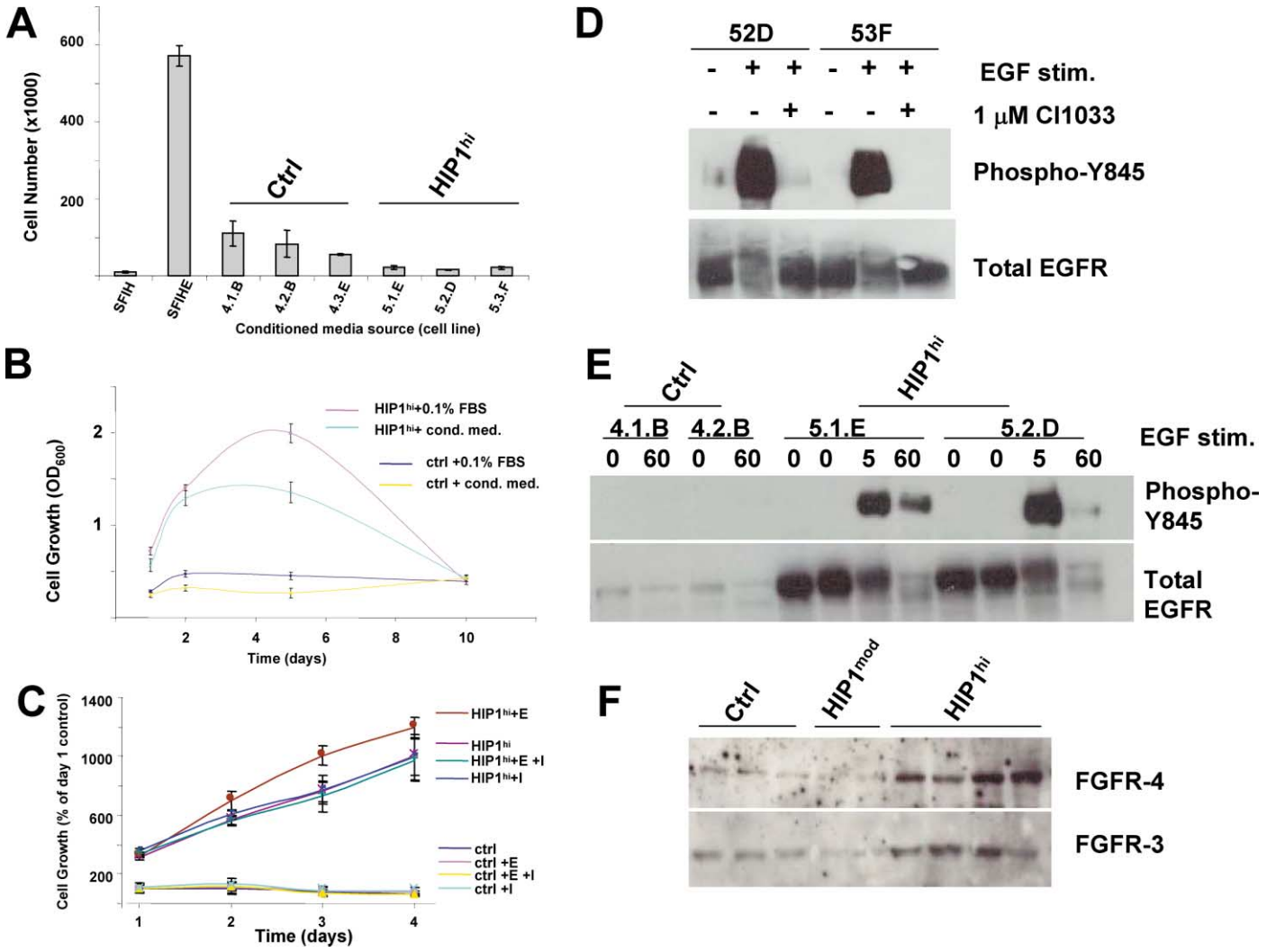

Figure 7. Analysis of requirement of EGF for serum-independent growth and EGFR activation in 3T3/HIP1^{hi} cell lines

A: Secretion of factors with EGF-like activity by 3T3/HIP1^{hi} cells was assayed by growing MCF10A cells in SFIH medium supplemented with tissue culture supernatant from 3T3/HIP1^{hi} cells. SFIH, serum-free medium supplemented with insulin and hydrocortisone; SFIHE, SFIH medium supplemented with EGF. Results are mean MCF10A cell number ± standard deviation after seven days of growth in the indicated media.

B: Cell growth in conditioned medium from 3T3/HIP1^{hi} cells. Pink: 3T3/HIP1^{hi} cells + 1% FBS; light blue: 3T3/HIP1^{hi} cells+ 3T3/HIP1^{hi} conditioned medium; dark blue: 3T3/Ctrl + 1% FBS; yellow: 3T3/Crl + 3T3/HIP1^{hi} conditioned medium.

C: EGF stimulates the growth of 3T3/HIP1^{hi} cells in 0.1% FBS, and EGFR inhibition reduces growth of 3T3/HIP1^{hi} cells in 0.1% FBS plus EGF. All cells were grown in 0.1% FBS with the daily addition of EGF (100 ng/ml) and/or CI-1033 (1 μM). E = EGF. I = EGFR inhibitor. The *y* axis represents percent of the OD600 at day 1 of the Ctrl cells with nothing added to the 0.1% FBS-containing media.

D: Stimulation of EGFR with EGF and inhibition by CI1033. 3T3/HIP1^{hi} cells were starved in serum-free medium and then stimulated with either EGF at 100 ng/ml with or without pretreatment for 1 hr with 1 μM CI1033 as indicated. 50 μg of protein were analyzed by Western blot for phosphorylated EGFR and total EGFR as indicated.

E: EGF stimulation of 3T3/Ctrl and 3T3/HIP1^{hi} cells. 50 μg of protein were analyzed with antibodies to phosphorylated and total EGFR.

F: Upregulation of other tyrosine kinase receptors in 3T3/HIP1^{hi} cells. 50 μg of protein extracts from the stably transfected cell lines were analyzed by Western blot for FGFR-3 and FGFR-4 as indicated.

that there was not significant EGF-like activity secreted by the transformed cells into the conditioned medium. Thus, the conditioned medium from the HIP1-transformed cells did not contain growth factors that could stimulate either NIH/3T3 cells or MCF10A cells to grow.

Since there was no added activity in the conditioned medium from the HIP1-transformed cells, and since the cells required FBS to grow in soft agar, we next tested if EGFR activation in 3T3/HIP1^{hi} cells was constitutive, or whether it required stimulation by EGF-like compounds. Following starvation of serum, we found that the EGFR in 3T3/HIP1^{hi} cells was quiescent, and its activation (tyrosine phosphorylation) was very sensitive to stimulation by EGF (Figures 7D and 7E). This stimulation was completely inhibited by the EGFR inhibitor, CI-1033 (Figure 7D) (Slichenmyer and Fry, 2001). When 3T3/HIP1^{hi} cells were grown in medium containing the EGFR inhibitor CI-1033, there was inhibition of EGF-stimulated growth in 0.1% FBS (Figure 7C) and inhibition of their ability to form foci when plated at low

density in 10% or 0.1% FBS. When CI-1033 was added, ability of HIP1^{hi} cells to form colonies in 0.1% FBS was completely inhibited, while the ability to form colonies in 10% FBS was significantly inhibited with decreases in colony number as well as colony size (Figure 5D). Addition of EGF to HIP1^{hi} cells plated in 0.1% FBS caused an increase in the number of colonies formed, and this effect was abrogated by the addition of CI-1033 (Figure 5D). As expected, when plated at low density, 3T3/Ctrl cells that were not expressing HIP1 did not form significant numbers of colonies in 10% FBS and 0.1% FBS. These data demonstrated that EGF/EGFR were required for some aspects of the transformed phenotype. However, the lack of a large effect of the inhibitor on growth per se (Figure 7C) indicated that other aspects of the transformed phenotype occurred as a result of more widespread effects of HIP1.

To begin to test what these additional effects might be, we assessed the levels of expression of other candidate growth factor receptors in the HIP1-transformed cells compared to the control cells. We found that FGFR-3 and FGFR-4 were consistently upregulated in 3T3/HIP1^{hi} cells (Figure 7F). Thus, the growth of the HIP1-transformed cells in low serum or at confluence was not solely dependent on EGFR activity and may be the result of upregulation of other tyrosine kinase receptors as well. In contrast, the focus formation assay that is a bona fide measure of transformation was dependent on active EGF signaling pathways.

Because a major abnormality in these cells was the increased level of multiple growth factor receptors, we began to investigate how HIP1 might cause the upregulation of these receptors. Since HIP1 physically interacts with clathrin and the endocytic adaptor protein, AP2, the effect of HIP1 on the levels and localization of these proteins was examined. In 3T3/HIP1^{hi} cells, there was no change in the overall levels of clathrin heavy chain, but AP2 levels were diminished (Figure 8A). In addition, the subcellular localization of clathrin and AP2 in 3T3/HIP1^{hi} cells and the 3T3/Ctrl cells were assessed. In the 3T3/HIP1^{hi} cells, location of the remaining AP2 was not altered compared to the control cells (data not shown). In contrast, clathrin was concentrated in the perinuclear area, with less clathrin present at the cell periphery compared to the 3T3/Ctrl cells (Figure 8B). Coimmunofluorescent labeling with a Golgi marker and clathrin confirmed that clathrin was concentrated in the area of the trans-Golgi network (Figure 8C). This Golgi localization suggests that in these HIP1-transformed cells, clathrin is less available to participate in endocytosis at the cell membrane. The decreased levels of AP2 may explain this altered localization, as AP2 normally recruits clathrin to the plasma membrane. This mislocalization of clathrin could cause an attenuation of normal endocytosis and decrease the steady state degradation of growth factor receptors, in turn leading to an increase in the overall level of receptor tyrosine kinases. This also explains why the HIP1-transformed cells have increased sensitivity to growth factor stimulation.

The alteration of trafficking in these cells was also examined by immunofluorescent and flow cytometric assays to assess EGF and transferrin accumulation in cells. Prior studies have shown that expression of HIP1 mutants lacking the ENTH and coiled-coil domains are potent inhibitors of endocytosis of transferrin, while the full-length protein promoted efficient uptake (Metzler et al., 2001). First, the effect of HIP1 overexpression on EGF trafficking was examined. This analysis demonstrated that EGF accumulation in the cell was dramatically increased in the 3T3/HIP1^{hi} cells (Figure 8D). We also found that accumulation of transferrin was increased in HIP1-overexpressing cells compared to 3T3/Ctrl cells. This accumulation was assessed both by confocal microscopy (Figure 8E) and flow-cytometry (Figure 8F). The absence of such an effect on transferrin accumulation in vector-transfected cells demonstrated that this effect was specific to HIP1 overexpression. As one might expect, 3T3/HIP1^{mod} cell lines had intermediate levels of transferrin and EGF uptake (data not shown). Hence, HIP1 led to increased levels of receptors in the HIP1-transformed cells, and this led to increased ligand accumulation and activation of receptors.

Since EGFR is often overexpressed in breast cancer and EGFR was upregulated in the HIP1-transformed cells, we sought to obtain a concomitant analysis of HIP1 and EGFR expression in a series of primary human breast tumors. Using immunohistochemistry and tissue microarrays (TMAs), a comprehensive analysis of HIP1 and EGFR expression was achieved in breast cancer. 183 specimens from 40 patients with breast cancer were represented on a TMA that included sections corresponding to normal breast, fibrocystic changes, atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS), and invasive breast cancer. The array was first analyzed for HIP1 expression by immunohistochemical staining. Normal breast tissue mostly exhibited low or no HIP1 expression (Figure 9A), as did breast tissue with fibrocystic changes (data not shown). ADH and DCIS, which are noninvasive lesions thought to be precursors of invasive breast cancer, had mostly moderate and high levels of HIP1 expression (Figures 9B–9D). Invasive carcinomas also tended to have moderate and high levels of HIP1 expression (Figures 9E and 9G), although a minority of carcinomas did not have any HIP1 expression (Figure 9F). These differences in HIP1 expression between normal and neoplastic tissue in this group of patients were highly significant (Figure 9H; Pearson's chi-squared, $p < 0.001$). Additionally, comparison of tissue samples within each patient showed higher levels of HIP1 expression in cancerous as opposed to normal tissue (paired sample t test, $p < 0.001$; Supplemental Figure S1 at http://www.cancercell.org/cgi/content/full/3/5/471/DC1). Thus, elevated expression of HIP1 marked neoplastic tissue within clinical specimens that contained both normal and cancerous tissues. The elevated levels of HIP1 in cancerous tissue led us to stain the same TMA for EGFR to determine if, in vivo, HIP1 expression led to the same elevation of the EGFR seen in 3T3-HIP1^{hi} cell lines. In fact, we found that tumors that did not express HIP1 did not express EGFR (Figure 9I), while tumors that overexpressed HIP1 also overexpressed EGFR (Figure 9J).

Consistent with the elevated expression of HIP1 seen in primary tumors, we have also found that all the breast cancer cell lines we have tested expressed high levels of HIP1 (Rao et al., 2002). Since HIP1 overexpression led to oncogenic transformation, we tested the ability of a dominant interfering mutant, HIP1/ΔE, to induce apoptosis in the HIP1-expressing breast cancer cell line BT549. Indeed, overexpression of the HIP1/ΔE led to apoptosis in this cell line as assayed by TUNEL (Supplemental Figure S2 at http://www.cancercell.org/cgi/content/full/3/5/471/DC1). This analysis, together with the cumulative data on HIP1 overexpression in primary tumors and HIP1's ability to confer transformation properties on NIH 3T3 cells, suggests that HIP1 is a novel therapeutic target.

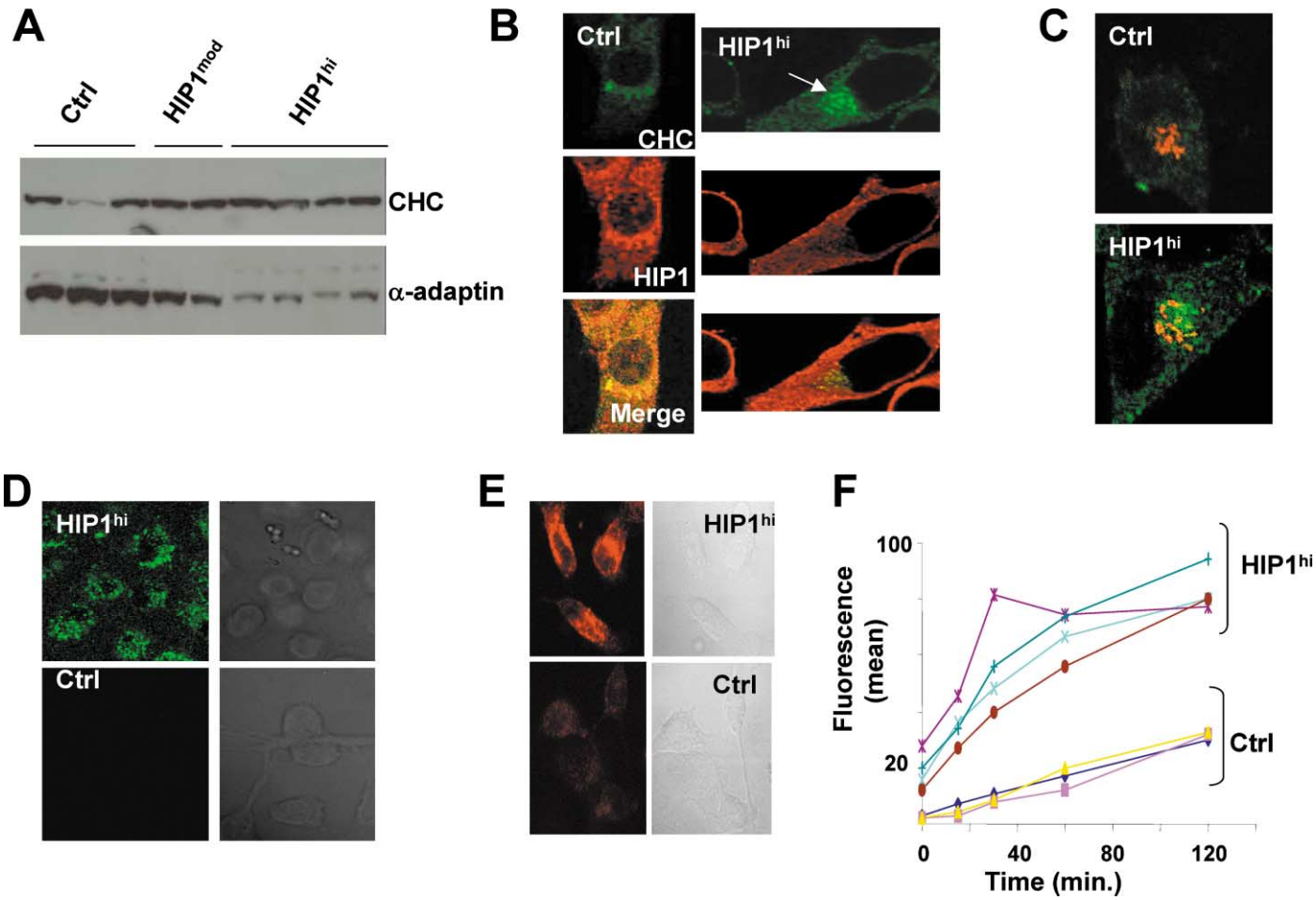

Figure 8. Alterations in endocytosis in HIP1-overexpressing cells

A: Western blot analysis of levels of endocytic HIP1 interactors in stable cell lines used in this study. CHC, clathrin heavy chain; α-adaptin is the 110 kDa subunit of the AP2 adaptor protein complex.

B: Confocal immunofluorescent analysis of HIP1 and clathrin heavy chain (CHC) in 3T3/Ctrl and 3T3/HIP1^{hi} cells. Cells were coimmunostained for CHC (green) and HIP1 (red).

C: Confocal immunofluorescent analysis of CHC (green) and the Golgi marker, GM130 (red). The merged images are shown to demarcate areas of colocalization (yellow).

D: Stably transfected cells were plated on coverslips, serum-starved, incubated for 60 min at 4°C with FITC-EGF (molecular probes), and then shifted to 37°C for 30 min, fixed, and mounted onto slides. Images were taken on Zeiss LSM confocal microscope. The 3T3/Ctrl and 3T3/HIP1^{hi} cell lines were analyzed for uptake at the same time and the camera's exposure time for visualization of the uptake was identical. The photos are representative of what was visualized for the 3T3/HIP1^{hi} (upper panels) and the control lines (lower panels).

E: Images of cell lines following AlexaFluor 633-transferrin uptake. The camera's exposure time for visualization of the uptake was identical as described in **D**.

F: AlexaFluor 633-conjugated transferrin accumulation in NIH 3T3 stable lines as quantitated by flow cytometry. The cell lines tested in this assay were vector control (3T3/Ctrl) lines (3T3/4.1.B, 4.2.B, 4.3.E) and high-HIP1 expressing lines (3T3/5.1.E, 5.2.C, 5.2.D, and 5.3.F). To allow for binding of transferrin to the transferrin receptor, all the samples were kept at 4°C for 1 hr after addition of AlexaFluor 633-transferrin. Cells were then transferred to 37°C and aliquots were analyzed by flow cytometry at various time points. Each time point was done in triplicate and the averages are presented. Three independent experiments were performed on all cell lines.

Discussion

Our data, which indicate a fairly global effect on endocytosis in 3T3/HIP1^{hi} cell lines, including changes in AP2 levels, a redistribution of clathrin, and elevated levels of multiple cell surface receptors, suggest that HIP1's rate-limiting role in clathrin-mediated trafficking is the mechanism of transformation. Additional evidence for a role of endocytic proteins in cellular transformation comes from studies of Eps15, a protein that colocalizes with clathrin-coated vesicles (Confalonieri et al., 2000). Overexpression of Eps15 in NIH/3T3 fibroblasts causes anchorage-independent growth, similar to what is reported here for HIP1 (Fazioli et al., 1993). A second endocytic protein, intersectin, has also been shown to cause anchorage-independent growth in NIH/3T3 fibroblasts when overexpressed (Adams et al., 2000). Whether the Eps15 and intersectin proteins are elevated in primary human cancers, cause cells to form tumors in vivo, or cause cells to grow in the presence of limiting serum concentrations remains to be determined.

As mentioned above, the mechanism of HIP1-mediated

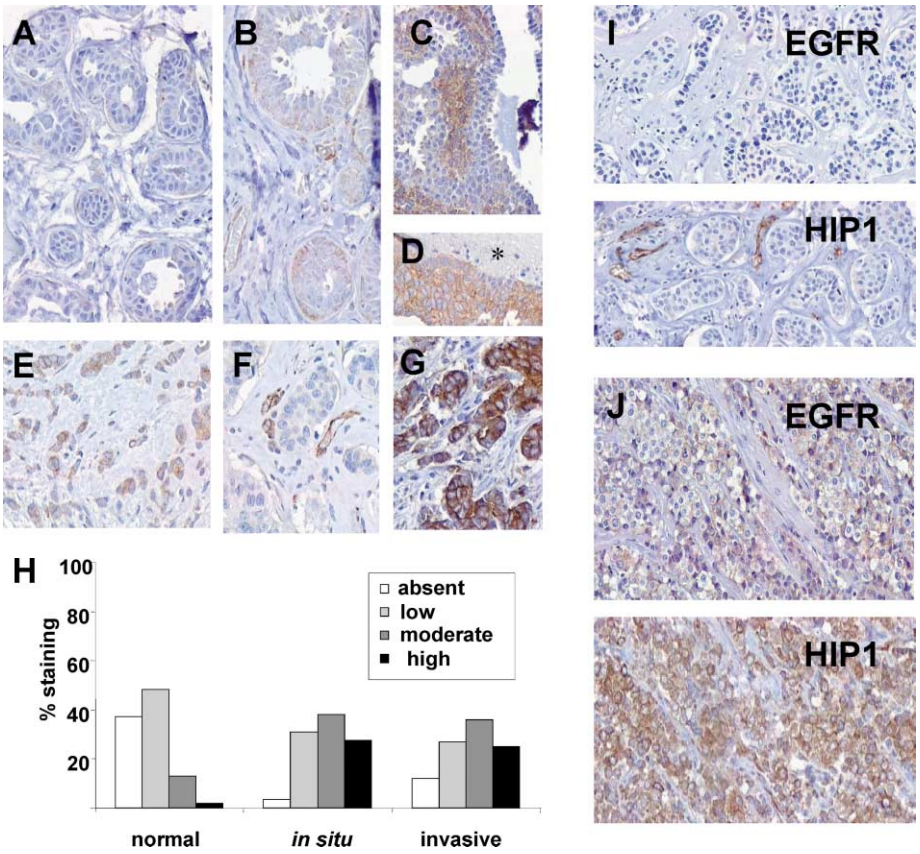

Figure 9. HIP1 and EGFR expression in primary human breast tissues

Slides were stained with monoclonal anti-HIP1 antibody 4B10 or the EGFR antibody (Zymed Laboratories Inc). Slides were reviewed in their entirety during three independent reading sessions by a pathologist (C.G.K.), an oncologist (T.S.R.), and two of the other authors (D.S.R., T.S.H.). There was greater than 95% concordance of score assignments between the three independent reading sessions. **A–G:** Low-power photomicrographs of representative specimens: normal breast epithelium **(A)**; ADH with HIP1 staining in myoepithelial cells **(B)**; papillary hyperplasia with HIP1 staining of the endothelium in the fibrovascular core **(C)**; HIP1 staining of DCIS with comedo necrosis (*) **(D)**; HIP1 staining of invasive lobular carcinoma with typical single filing of tumor cells **(E)**; invasive ductal carcinoma with no HIP1 staining **(F)**; and invasive ductal carcinoma with high HIP1 staining **(G)**.

H: Histogram of immunohistochemical score distribution (HIP1 staining) from analysis of breast tissue samples. 183 specimens from 40 patients were analyzed. Samples were grouped into three categories (normal, in situ, invasive). The normal category included samples diagnosed as normal and fibrocystic changes, the in situ category consisted of atypical ductal hyperplasia and DCIS, and the invasive category consisted of both invasive lobular and ductal carcinoma.

I: EGFR staining is negative in HIP1-negative primary breast tumors.

J: HIP1 is overexpressed in EGFR-positive tumors.

transformation of NIH/3T3 cells likely involves a profound dysregulation of trafficking of multiple growth factor receptors. Previous published results suggest that HIP1 promotes endocytic uptake of cell surface receptors (Metzler et al., 2001). With the latter observations, it may not be obvious why endocytosed receptors became upregulated rather than downregulated in the HIP1-transformed cells. One possibility is that HIP1 is rate-limiting to the process of endocytosis but also inhibits lysosomal targeting and degradation of the receptors, leading to increased recycling of these receptors to the cell surface. Overexpression of another Huntingtin associated protein, HAP1, has also recently been shown to inhibit the degradation of EGFR (Li et al., 2002). Alternatively, HIP1, by virtue of its ability to bind to AP2, may lead to AP2 downregulation posttranscriptionally. This in turn could cause clathrin to be redistributed to the trans-Golgi network (as AP2 is required for recruitment of clathrin to the cell surface), where it is less available for endocytosis. This would then lead to cell surface receptor upregulation and, because of the increased receptor density, increased endocytosis upon ligand stimulation. In addition to these proposed mechanisms for HIP1-mediated upregulation of cell surface receptors, there is data to suggest that full intracellular signaling originating from receptor tyrosine kinases requires the ligand-mediated endocytosis of these receptors (Vieira et al., 1996). Since uptake of EGF was increased in HIP1-transformed cells, this may contribute to the increased levels of mitogenic signaling observed in these cells.

This dysregulation of endocytosis caused a change in the steady-state levels of multiple cell surface receptors, including the EGFR. The increase in surface levels of growth factor receptors would be expected to lead to the observed increased sensitivity of the cells to growth factors. We speculate that EGFR upregulation may be a major contributing mechanism toward transformation. The main reason for this is that the overexpression of EGFR has been shown previously to be a transforming event in NIH/3T3 cells in an EGF-dependent manner (Di Fiore et al., 1987a), and the transformed phenotype in our case required the presence of extrinsic growth factors. However, the initial event of receptor tyrosine kinase overexpression and increased activation by the overexpression of HIP1 may have then allowed further mutations to accumulate, leading to the phenotype of growth in lowered serum that was independent of EGF stimulation (Figure 7C). The phenomenon of an initial oncogenic stimulus being no longer required for the perpetuation of some aspects of the transformed phenotype was recently described elegantly in a mouse model system (Moody et al., 2002). Alternatively, the dysregulation of trafficking of multiple GFRs may explain the partial dependence on the EGFR for transformation.

In sum, the ability of HIP1 to transform cells is consistent with its overexpression in breast, colon, and prostate cancer as well as multiple other cancers (Rao et al., 2002). It is also consistent with the idea that HIP1 expression is necessary for the survival of some cells (Rao et al., 2002). This report directly links the altered expression in epithelial cancers of a protein involved in clathrin trafficking with known pathways of cellular transformation. It does this by showing that HIP1-mediated transformation results from altered clathrin trafficking that leads

to elevated levels and activation of receptor tyrosine kinases. HIP1 could therefore be both an important tumor marker and a novel target for therapy. By linking the basic biological process of trafficking and mitogenic intracellular signaling with oncogenesis, a new insight has been gained into the mechanisms whereby normal cells become cancerous.

Experimental procedures

Generation of cell lines overexpressing HIP1

The construction of the full-length HIP1 construct, pcDNA3/FLHIP1, has been described (Rao et al., 2002). The insert, containing the entire HIP1 coding sequence as well as 60 base pairs of 5′ untranslated DNA in exon 1, was subcloned into pTRE2-Hyg (Clontech). Ha-RasV12 was excised with BamHI (5′) and EcoRI (3′) from pBabepuro/Ha-RasV12 (kind gift of Dr. Maria Soengas, Cold Spring Harbor Laboratories) and subcloned into the BamH1/EcoRI-digested pcDNA3 to create pcDNA3/Ha-RasV12. NIH/3T3 (ATCC, Manassas, VA) cells were transfected with pTRE2-Hyg or pTRE2-Hyg/HIP1 and selected in 200 μg/ml Hygromycin B (Clontech), or pcDNA3/Ha-RasG12V, and selected in 400 μg/ml G418 (Invitrogen). Individual clones were picked after 2 weeks of selection, and cell lines were passaged every four days. All clones from transfections with HIP1 and RasV12 demonstrated expression of HIP1 and Ras respectively. All experiments were performed with cell lines at the same passage number.

Western blot analyses

Cells were harvested at confluence and lysed in Lysis Buffer A[10], supplemented with protease/phosphatase inhibitors (Roche), run on 7% or 10% SDS-PAGE transferred to nitrocellulose, and immunoblotted with the following antibodies: HIP1/4B10, which recognizes human HIP1 only, and HIP1/1B11, which recognizes both mouse and human HIP1. The anti-clathrin monoclonal antibody, Td.1, was the kind gift of Linton Traub, University of Pittsburgh. Other antibodies were commercially available. (Anti-α-adaptin monoclonal antibody, Sigma Chemical Company, St. Louis, MO; Ras monoclonal antibody, Transduction Labs, San Diego, CA; phospho ERK 1/2, FGFR-3, and FGFR-4, Santa Cruz Biotechnology, Santa Cruz, CA; all other antibodies were obtained from Cell Signaling Technologies, Beverly, MA).

Low-density focus formation in limiting serum

For plating at low density in limiting serum, the method of Sage et al. (2000) was modified as follows. Cells were briefly trypsinized and resuspended in DMEM supplemented with 0.1% FBS. 1,300 cells were immediately plated in the same medium. The next day, the medium was changed to include either EGF (100 ng/ml) and/or the erbB inhibitor, CI-1033 (1 μM). The medium was changed every third day. At day 10, the plates were examined for the presence of colonies visible to the naked eye and quantitated.

Analysis of HIP1 and EGFR expression in primary breast tumors

TMAs were created as previously described (Kononen et al., 1998; Perrone et al., 2000). Cases of breast cancer were collected from sequential patients seen at the University of Michigan breast care center. Staining for HIP1 was as described (Rao et al., 2002). Initial sections were stained for H and E to verify histological diagnoses. Statistical analysis was completed using the SPSS program as previously described (Rao et al., 2002).

Apoptosis assay

Apoptosis was determined by transfecting the BT549 human breast cancer cell line with full-length HIP1 and HIP1/ΔE. After allowing for expression of the protein (15 hr), cells were fixed in PBS with 5% paraformaldehyde for 1 hr and permeabilized with 0.1% Triton X-100. Cells were then labeled with fluorescein-tagged dUTP using the InSitu Cell Death Kit (Roche Molecular Biochemicals), following manufacturer's instructions. Following TUNEL labeling, cells were labeled with HIP1/4B10, followed by Texas Red-tagged secondary antibody. Labeling was visualized by confocal microscopy (Carl Zeiss MicroImaging Inc., Thornwood, New York) as previously described (Rao et al., 2001). The proportion of transfected cells (labeled red) that were also TUNEL-positive (green) was counted.

Immunofluorescent labeling and confocal microscopy

For immunofluorescent labeling of cells, the polyclonal HIP1 antibody, pc-HIP1, the monoclonal anti-clathrin antibody, X22 (kind gift of Dr. Linton Traub, University of Pittsburgh), and the Golgi marker, GM130 (Signal Transduction Labs) were utilized as previously described (Rao et al., 2001, 2002). Images were acquired under Zeiss LSM 510 confocal microscope as previously described, and images were processed utilizing Adobe Photoshop.

Analysis of EGF and transferrin trafficking

For immunofluorescent analysis of transferrin and EGF uptake, stably transfected cells were plated on coverslips so that they were about 70% confluent the day of the experiment. Cells were then serum-starved for 3 hr prior to addition of the labeled transferrin or 16 hr prior to addition of EGF. AlexaFluor 633-transferrin or FITC-EGF (Molecular Probes) was added to the cells at 50 μg/ml in DMEM, and incubated at 4°C for 60 min. The cells were then incubated at 37°C for 30 min, fixed in 3% formaldehyde, washed with PBS, and mounted onto slides with Vectashield. Images (fluorescent and Nomarsky) were taken on Zeiss LSM confocal microscope.

For analysis of Alexa Fluor 633-transferrin accumulation by flow cytometry, confluent cells were serum-starved for 3 hr, after which they were trypsinized. They were then washed in cold PBS/1%BSA twice and resuspended in DMEM. Alexa Fluor 633-Transferrin (Molecular Probes) was added (50 μg/ml) to the cells on ice. The cells were then maintained, rotating in the dark at 4°C for 1 hr. To test for uptake, the cells were shifted to 37°C and maintained in the dark, rotating. At each timepoint, cells were washed in PBS at 4°C and resuspended in 1% paraformaldehyde in PBS. The samples were then analyzed for fluorescence by flow cytometry.

Preparation of and use of conditioned media from 3T3/HIP1^{hi} cell lines

3T3/HIP1^{hi} cell lines were grown in medium supplemented with 1% FBS for 48 hr, and the tissue culture supernatant was collected after cellular debris was removed by centrifugation. This medium was then used to supplement SFIH medium in a 1:1 ratio, in order to grow MCF10A cells (which are EGF-dependent). Briefly, 3.5×10^4 MCF10A cells were plated in triplicate in six-well dishes, and grown in medium for 7 days as described (Ethier et al., 1991). Medium was replaced every two days. Cell numbers were quantitated with a coulter counter. Cells grown in SFIH medium supplemented with EGF (SFIHE) were the positive control.

3T3/Ctrl and 3T3/HIP1^{hi} cells were grown in medium supplemented with conditioned medium from the 3T3/HIP1^{hi} cells to determine if secreted factor(s) was responsible for the increased growth properties of 3T3/HIP1^{hi} cells. Cell lines were plated in 96 well plates at a concentration of 5×10^4 cell/ml in 100 μl per well of DMEM media supplemented with 1% FBS. 100 μl of conditioned media from 3T3/HIP1^{hi} cell lines was added 24 hr later, and the negative control cells received 100 μl of medium supplemented with 1% FBS. MTT reagents were added at days 1, 2, 5, and 10, according to manufacturer's protocol (Roche), and the plate was read at a wavelength of 600 nm. Results from cells treated with medium supplemented with 1% FBS are an average of 12 wells per day with 3 cell lines represented. Results from cells treated with conditioned media are an average of 36 wells per day with 3 cell lines and 3 types of conditioned media represented.

EGFR inhibitor effect on the MTT assay

Cell lines were plated in 96-well plates at a concentration of 5×10^4 cell/ml in DMEM media with 0.1% FBS. EGF inhibitor CI1033 (1 μM) and /or EGF (100 ng/ml) was added daily, starting 24 hr after plating the cells. MTT reagents were added at days 1, 2, 3, and 4, according to manufacturer's protocol (Roche), and the plate was read at a wavelength of 600 nm. Results are an average of 12 wells per day with 3 cell lines represented.

EGF stimulation of cell lines

3T3/HIP1^{hi} cells were plated in 6-well plates or 150 mm dishes. 24 hr later, cells were starved in serum-free media. After 4 hr of serum starvation, cells were incubated for 1 hr with CI-1033 and then treated with 100 ng/ml EGF for 5 min and lysed. 50 μg of protein from each lysate was analyzed by Western blot. Membranes were immunoblotted with either polyclonal anti-phospho-EGFR (Tyr845) antibody or polyclonal anti-EGFR antibody (both from Cell Signaling, Inc.).

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